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ECBC-TR-1402

IMMOBILIZATION OF ORGANOPHOSPHORUS ACID ANHYDROLASE MUTANT Y212F ON SILICA NANOSPHERES

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September 2016

U.S. ARMY RDECOM

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE ***(DD-MM-YYYY)***	**2. REPORT TYPE**	**3. DATES COVERED** ***(From - To)***
XX-09-2016	Final	Aug 2014 – Dec 2014

4. TITLE AND SUBTITLE
Immobilization of Organophosphorus Acid Anhydrolase Mutant Y212F on Silica Nanospheres

5a. CONTRACT NUMBER

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER
R.0014307.133.1

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
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nanoComposix; 4878 Ronson Court, Suite K; San Diego, CA 92111-1806

8. PERFORMING ORGANIZATION REPORT NUMBER
ECBC-TR-1402

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Defense Threat Reduction Agency; 8725 John J Kingman Road #6201; Fort Belvoir, VA 22060-6201

10. SPONSOR/MONITOR'S ACRONYM(S)
DTRA

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution is unlimited.

13. SUPPLEMENTARY NOTES

14. ABSTRACT-LIMIT 200
We have engineered mutants of the wild type organophosphorus acid anhydrolase (OPAA) for activity on G-type chemical nerve agents and observed increased activity on the most-toxic enantiomers. Enzyme stability might be enhanced by attachment of the OPAA to a silicon dioxide nanoparticle. Conjugation of our enzyme mutant was performed by personnel at nanoComposix (San Diego, CA), which is a producer of customized nanoparticles. After conjugation, the activity of the enzyme–particle combination on pinacolyl methyl phosphonofluoridate (GD, soman) was measured and compared with free enzyme. Activity measurements were made to compare a free and immobilized OPAA mutant on the G-agent stimulant diisopropyl fluorophosphates (DFP) and soman.

15. SUBJECT TERMS
Organophosphorus acid anhydrolase (OPAA) Enzyme-conjugated nanoparticle Enzymatic activity on soman
Pinacolyl methyl phosphonofluoridate, soman (GD) Diisopropyl fluorophosphate (DFP)

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			Renu B. Rastogi
U	U	U	UU	22	**19b. TELEPHONE NUMBER** ***(include area code)*** (410) 436-7545

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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PREFACE

The work described in this report was authorized under project no. R.0014307.133.1. This work was started in August 2014 and completed in December 2014.

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Acknowledgment

The Defense Threat Reduction Agency is gratefully acknowledged for sponsorship of this work.

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IMMOBILIZATION OF ORGANOPHOSPHORUS ACID ANHYDROLASE MUTANT Y212F ON SILICA NANOSPHERES

1. INTRODUCTION

Catalytic enzymes have the potential to protect humans against organophosphate poisoning, if they are administered before exposure to the contaminant, and they could be used to detoxify these compounds after exposure, if a therapeutic compound can be developed. In addition to effectiveness, a successful therapeutic compound would need to have a sufficient shelf life in liquid or dry form and be thermally stable. Stability and shelf life might be increased by attaching the compound to other particles, including silicon dioxide.

The purpose of this study was to determine if an enzyme that is shown to have good activity on an organophosphorus nerve agent can retain this activity and to determine at what level this activity can be retained after attachment of the enzyme to a silicon dioxide nanoparticle.

The enzyme Y212F is an engineered mutant of the organophosphorus acid anhydrolase (OPAA) with good catalytic activity on diisopropyl fluorophosphate (DFP) and pinacolyl methyl phosphonofluoridate (GD, soman). This enzyme was used as a baseline to measure changes in immobilized enzyme activity. DFP was used during binding optimization studies at nanoComposix (San Diego, CA). GD was used as the substrate at the U.S. Army Edgewood Chemical Biological Center (ECBC) to measure enzyme activity before and after stabilization on the silica nanoparticle.

2. METHODS

2.1 Production of Y212F Enzyme

The OPAA Y212F *Escherichia coli* clone was prepared by DNA2.0, Inc. (Menlo Park, CA) personnel with the amino acid sequence shown in Figure 1.

```
MNKLAVLYAE HIATLQKRTR EIIERENLDG VVFHSGQAKR QFLDDMYYPF    50
KVNPQFKAWL PVIDNPHCWI VANGTDKPKL IFYRPVDFWH KVPDEPNEYW   100
ADYFDIELLV KPDQVEKLLP YDKARFAYIG EYLEVAQALG FELMNPEPVM   150
NFYHYHRAYK TQYELACMRE ANKIAVQGHK AARDAFFQGK SEFEIQQAYL   200
LATQHSENDT PFGNIVALNE NCAILHYTHF DRVAPATHRS FLIDAGANFN   250
GYAADITRTY DFTGEGEFAE LVATMKQHQI ALCNQLAPGK LYGELHLDCH   300
QRVAQTLSDF NIVNLSADEI VAKGITSTFF PHGLGHHIGL QVHDVGGFMA   350
DEQGAHQEPP EGHPFLRCTR KIEANQVFTI EPGLYFIDSL LGDLAATDNN   400
QHINWDKVAE LKPFGGIRIE DNIIVHEDSL ENMTRELELD
```

Figure 1. OPAA Y212F amino acid designations.

E. coli cell cultures were grown in Luria broth (Sigma-Aldrich Company; St. Louis, MO) with 100 µg/mL of ampicillin and 0.1 mM $MnCl_2$ at 25 °C to the late log or early stationary phase. Cells were separated using a centrifuge at 14,000 × *g* for 15 min. Cells were resuspended in 10 mM bis-trispropane (BTP) and 0.1 mM $MnCl_2$ at pH 7.2 at 3 mL/g of pellet weight. Cells were lysed with a sonic nozzle using three 15 s bursts in an ice bath. Cellular debris was separated at 18,000 × *g* for 120 min. The active fraction was separated at 4 °C using an ÄTKA Express purification system (GE Healthcare Life Sciences; Uppsala, Sweden) with a Q-sepharose column (HiPrep Q FF 16/10; GE Healthcare Life Sciences) using a salt gradient of 0–0.4 M NaCl and a pH gradient of 7.2 to 6.8. The active peak was dialyzed in 10 mM BTP and 0.1 mM $MnCl_2$ at pH 7.2.

2.2 Conjugation of Y212F Enzyme to 80 nm Amine Terminated SiO_2

The 80 nm amine terminated SiO_2 particles (nanoComposix lot no. DAC1260) were previously fabricated at nanoComposix as a standard product (Figure 2). Before the material was used for conjugation, a quality check for size was performed using dynamic light-scattering and transmission electron microscopy (TEM), and a zeta potential was performed to check amine functionality. The conjugation of the OPAA mutant Y212F enzyme to the 80 nm amine SiO_2 (ID no. CLF0293) was performed following standard Solulink (Solulink, Inc.; San Diego, CA) conjugation chemistry. To perform this conjugation, a covalent bond was formed between an aromatic hydrazine, S-HyNic (succinimidyl 6-hydrazinonicotinate acetone hydrazone), and an aromatic aldehyde, S-4FB (succinimidyl 4-formylbenzoate), which were conjugated initially to amino groups on the enzyme and the particle of choice. To determine the success of the conjugation, the fluoride electrode assay was performed with the sample on DFP. Enzyme that was covalently bound to the SiO_2 particle as described had no DFP activity (data not shown).

A second conjugation of the Y212F enzyme to an 80 nm amine terminated SiO_2 with a longer linker was prepared following the same Solulink conjugation chemistry mentioned above for the initial conjugate (ID no. CLF0334). The longer linker, C6-S-4FB (C6-succinimidyl 4-formylbenzoate), has a chain of five carbons between the two reactive sites for conjugating free amino groups and S-HyNic. The longer linker was conjugated to the OPAA mutant Y212F enzyme with the shorter linker as previously described. A colorimetric bicinchoninic acid (BCA) assay was used to measure the enzyme bound to the particle for subsequent enzymatic activity determinations.

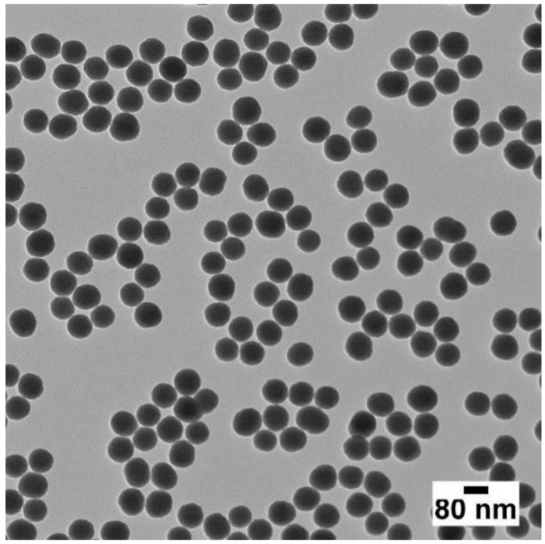
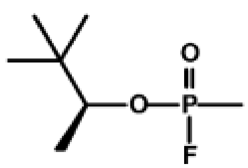


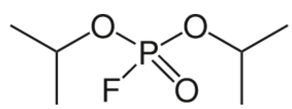
Figure 2. TEM image of 80 nm silica particles from DAC1260, which was employed for the conjugation of the Y212F enzyme.

2.3 Testing of Conjugated Enzyme Activity

The G-agent simulant DFP and agent GD, used in the study and represented in Figure 3, each contain fluoride that is released during hydrolysis. Enzymatic activity of these substrates can be measured by monitoring the fluoride concentration in solution after subtracting the spontaneous hydrolysis rate.



Soman (GD)



(DFP)

Figure 3. Chemical structures of GD and DFP.

G-agent substrates used in the study were Chemical Agent Standard Analytical Reference Material obtained from ECBC stocks.

Fluoride concentration was measured using a VWR International (Radnor, PA) Symphony probe and collected on a WTW (Wissenschaftlich-Technische Werkstätten GmbH; Weilheim, Germany) 7350 pH/Ion data logger. Assays were performed in a glass 10 mL double-wall reaction cell with circulating water at 25 °C. Activity was assayed in 50 mM BTP at pH 8.0

on a 3.0 mM substrate. Enzyme catalytic activity is expressed as the rate of fluoride ion production in micromoles per minute per milligrams of enzyme.

Sample concentrations for free enzyme in BTP solutions assayed at ECBC were measured as protein A280 using the Nanodrop 1000 software, version 3.8.1 (Thermo Fisher Scientific, Inc.; Waltham, MA). Nanospheres in suspension interfere with the Nanodrop A280 method; therefore, a BCA protein assay was used for conjugated enzyme concentration measurements. Enzyme concentration is expressed in milligrams per milliliter.

3. RESULTS

3.1 BCA Protein Data

Enzyme conjugated to the silica particles was measured as protein using the BCA protein method. Optical density (OD) was first calibrated against known protein concentrations, and a regression was calculated. The enzyme-conjugated particle sample was then measured to determine protein concentration. The BCA calibration data are presented in Figure 4 below.

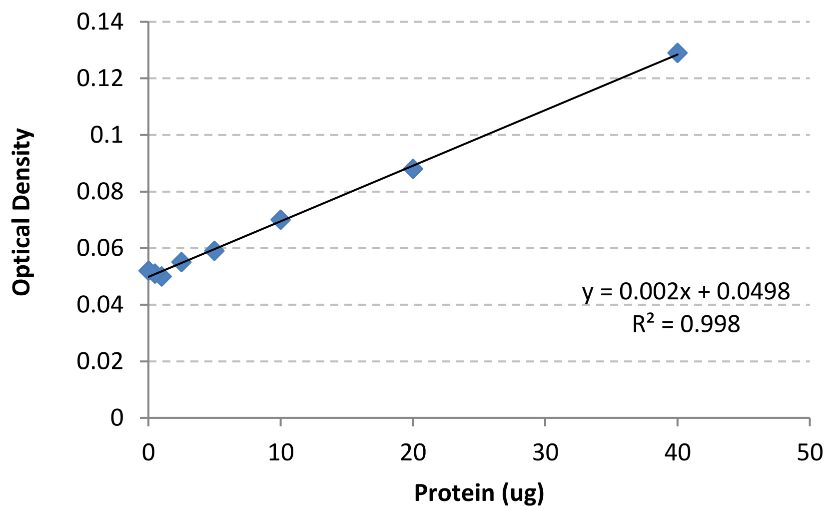


Figure 4. Graphed results from BCA calibration assay for eventual use in determining particle-binding efficiency and enzymatic activity.

In the conjugation methods described above, 1 mg of silica particles was combined with 50 µg of the OPAA mutant Y212F. The conjugated enzyme was washed multiple times to remove unbound enzyme. Enzyme-conjugated particle samples had an OD of 0.1175 for an average protein concentration of 33.85 µg/mL. The protein yield that was calculated from the BCA resulted in a protein yield of 67.7% of the initial amount.

Assuming all particles are spheres, and the density of silica is 2 mg/cm^3, the mass of a single 80 nm diameter silica particle can be calculated, yielding a total of 1.865×10^{12} particles in 1 mg of silica. Given a molecular weight for the enzyme of 50 kDa and a mass of 33.85 µg of enzyme per mg of silica, there are 4.06×10^{14} molecules of enzyme per mg of silica. This corresponds to 217 molecules of enzyme per 80 nm silica particle. If we assume that each enzyme is a sphere (potentially a gross simplification), the enzyme takes up a footprint on the surface of the particle related to the cross-sectional area of the circle defined by the radius of the sphere. For a spherical 50 kDa protein, the radius is thought to be 2.4 nm.[1] Thus, the area of the surface occupied by each enzyme is 18.1 nm^2. Therefore, the 217 enzyme molecules per particle will cover 3928 nm^2. Each particle has a surface area of 20,106 nm^2, which means that the percentage of enzyme-covered surface is around 19.5%.

3.2 Results on DFP

Fluoride release from the substrate was plotted versus time. The time points selected for measuring specific activity should be obtained from a linear portion of any curve. This is usually within the first 3 min of data collection. Fluoride concentration curves from the DFP assay are presented in Figure 5.

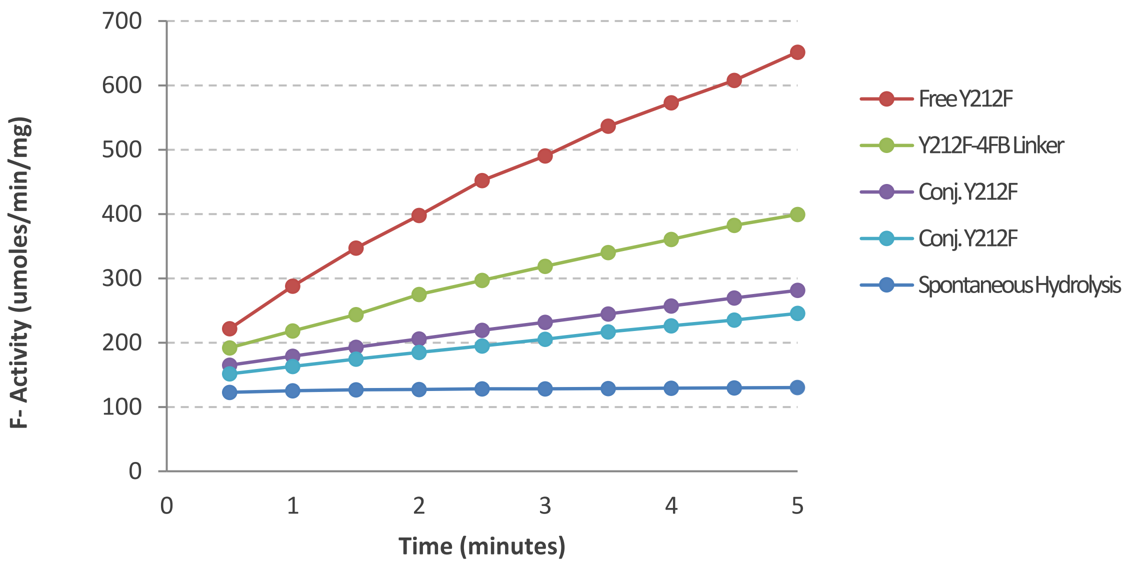


Figure 5. Fluoride assay results from three forms of the Y212F enzyme on DFP.

The results of fluoride ion probe testing on DFP, conducted by nanoComposix personnel, are shown in Table 1. Data points used for specific activity calculations were collected as soon as possible after enzyme addition and once the fluoride release had reached a linear response.

[1] Erickson, H.P. Size and Shape of Protein Molecules at the Nanometer Level Determined by Sedimentation, Gel Filtration, and Electron Microscopy. *Biol. Proced. Online* **2009,** *11*, 32–51.

Table 1. Results of Fluoride Release Assay from DFP Testing of Y212F Enzyme, before and after Conjugation to SiO_2 Particles

Substrate	DFP	DFP	DFP	DFP	DFP
Enzyme	No Enzyme	Free Y212F	Y212F-4FB Linker	Conj. Y212F	Conj. Y212F
µmol/min/mg	–	143.059	87.818	26.781	26.948
µmoles F-/min	0.002	0.251	0.154	0.063	0.050
µM F-/min	0.988	100.256	61.448	25.383	19.959
Enz start (mg/mL)	23.36	0.2336	0.714	0.03385	0.03385
Sub start (µmol/µL)	0.300	0.300	0.300	0.300	0.300
Enz (mg)	–	0.00175	0.00175	0.00237	0.00185
Enz/Particle (µL)	–	7.5	2.45	70	54.7
µL 0.3 M Sub	25	25	25	25	25
µM [substrate]	3000	3000	3000	3000	3000
Total assay vol (mL)	2.5	2.5	2.5	2.5	2.5
Spont t=1		126.65	126.65	126.65	126.65
Spont t=2		127.15	127.15	127.15	127.15
Enz t=1	126.65	346.97	243.51	192.81	174.26
Enz t=2	127.15	397.59	274.73	205.99	184.73
% [sub] hydroyzed	4.24	13.25	9.16	6.87	6.16
BTP (µL)	2450.00	2442.50	2447.55	2380.00	2395.30

Time (min)	Spontaneous Hydrolysis	Free Y212F	Y212F-4FB Linker	Conj. Y212F	Conj. Y212F
0.5	122.77	221.80	192.06	165.02	151.48
1.0	125.18	287.86	218.37	179.07	163.10
1.5	126.65	346.97	243.51	192.81	174.26
2.0	127.15	397.59	274.73	205.99	184.73
2.5	128.14	452.07	296.96	219.22	195.07
3.0	128.14	490.56	318.51	231.50	205.19
3.5	128.64	536.49	340.29	244.46	216.68
4.0	129.14	573.17	360.74	257.14	226.16
4.5	129.65	607.62	382.42	269.43	235.13
5.0	130.15	651.70	399.14	281.22	245.41

F-: fluoride ion; Conj.: conjugated; –: no data; Enz: enzyme; Sub: substrate; vol: volume; Spont: spontaneous; and t: time.

Table 2. Summary Results from Testing on DFP Substrate at nanoComposix

Parameter	Free Enzyme	Enzyme plus 4FB Linker	Conj. Y212F
Specific activity (µmol/min/mg)	143.06	87.82	26.78
	–	–	26.95
Specific activity average (µmol/min/mg)	–	–	26.86
DFP specific activity retained (%)	–	–	18.78

Conj: conjugated; –: no data.

3.3 Results on GD

Changes in fluoride concentration during the enzyme assays on GD are represented in Figure 6.

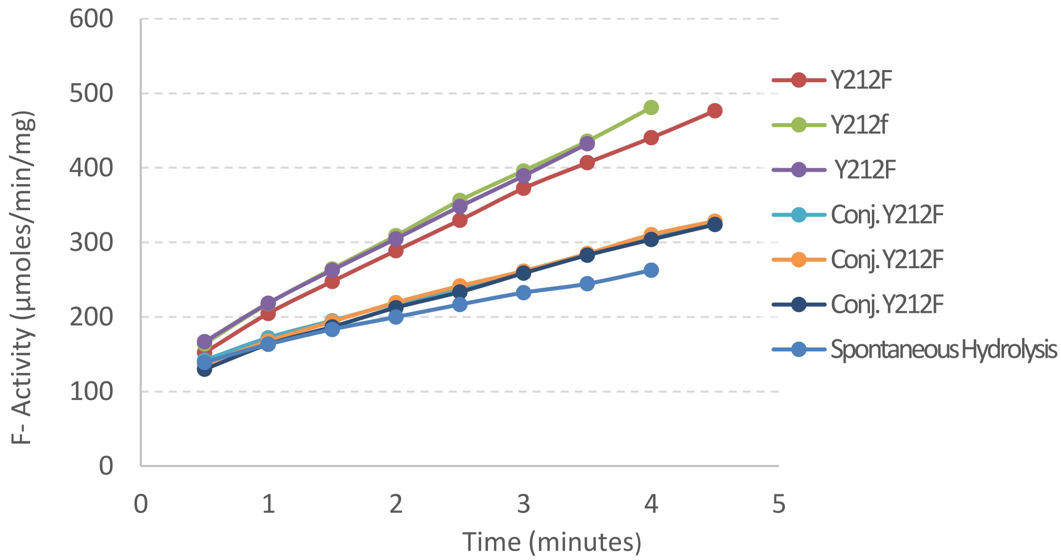


Figure 6. Graphical presentation of Y212F activity on GD.

The results of fluoride ion probe testing of conjugated enzyme on GD, conducted at ECBC, are displayed in Table 3. A summary of the results of specific activities on GD, which were retained after conjugation to SiO_2 particles, are listed in Table 4.

Table 3. Results of Fluoride Release Assay from GD Testing of Y212F Enzyme before and after Conjugation to SiO_2 Particles

Substrate	GD	GD	GD	GD	GD	GD
Enzyme	Y212F	Y212F	Y212F	Conj. Y212F	Conj. Y212F	Conj. Y212F
µmol/min/mg	284.67	317.85	298.59	41.36	46.00	40.99
µmoles F-/min	0.133	0.149	0.140	0.042	0.055	0.056
µM F-/min	53.200	59.400	55.800	16.800	21.800	22.200
Enz start (mg/mL)	0.2336	0.2336	0.2336	0.03385	0.03385	0.03385
Sub start (µmol/µL)	0.300	0.300	0.300	0.300	0.300	0.300
Enz (mg)	0.00047	0.00047	0.00047	0.00102	0.00118	0.00135
Enz/Particle (µL)	2	2	2	30	35	40
µL 0.3 M Sub	25	25	25	25	25	25
µM [substrate]	3000	3000	3000	3000	3000	3000
Total assay vol (mL)	2.5	2.5	2.5	2.5	2.5	2.5
Spont t=1	199.00	199.00	161.40	199.00	199.00	199.00
Spont t=2	213.80	213.80	175.70	213.80	213.80	213.80
Enz t=1	247.60	264.50	262.80	195.00	193.70	186.70
Enz t=2	289.00	309.00	305.00	218.20	219.40	212.60
% [sub] hydroyzed	9.633	10.300	10.167	7.273	7.313	7.087
BTP (µL)	2448.00	2448.00	2448.00	2420.00	2415.00	2410.00
Time (min)	Y212F	Y212F	Y212F	Conj. Y212F	Conj. Y212F	Conj. Y212F
0.5	152.10	164.00	166.70	141.90	136.40	129.80
1.0	204.80	218.00	218.60	172.20	167.70	163.40
1.5	247.60	264.50	262.80	195.00	193.70	186.70
2.0	289.00	309.00	305.00	218.20	219.40	212.60
2.5	329.80	356.20	348.10	238.80	241.50	233.50
3.0	372.50	396.10	389.30	261.10	261.60	258.70
3.5	406.90	435.90	432.60	283.40	285.00	282.90
4.0	440.40	481.00		306.40	310.40	303.90
4.5	476.50			324.30	328.50	324.00

F-: fluoride ion; Conj.: conjugated; –: no data; Enz: enzyme; Sub: substrate; vol: volume; Spont: spontaneous; and t: time.

Table 4. Summary of Retained Specific Activity of Enzyme Y212F on GD

Parameter	Y212F	Conj. Y212F
Specific activity (µmol/min/mg)	284.67	41.36
	317.85	46.00
	298.59	40.99
Specific activity average (µmol/min/mg)	300.37	42.78
GD specific activity retained (%)	NA	14.24

4. SUMMARY

Immobilizing an enzyme on a particle is a two-step process. The first step is to hybridize a linker to the enzyme, then conjugate the linker to the silica particle. The enzyme activity was measured by nanoComposix personnel on DFP attachment was started and after each step in the process. After completion, the immobilized enzyme activity was measured at ECBC using GD as a substrate.

In initial testing by nanoComposix personnel using DFP as a substrate, it was observed that when an initial short-linker was used, the result was very low retained activity, even after only the linker hybridization step. A longer linker was subsequently used, which resulted in better activity.

Data described herein indicates that although a significant percentage (67.7%) of the enzyme was successfully conjugated to the silica particles, only 14.24% of the activity on GD was retained. A loss in activity (38.6% loss) was observed at the 4FB linker hybridization step, and an additional loss occurred after the silica particle-conjugation step. Therefore, a large portion of the enzymatic activity was lost through conjugation to the particles. Ideally, there is a point of optimal protein-loading and linker length that can maintain activity. These must be measured against stability, shelf life, logistical burden, and effectiveness in a delivery system. More of these points need to be addressed as therapeutic development continues.

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ACRONYMS AND ABBREVIATIONS

BCA	bicinchoninic acid
BTP	bis-trispropane
C6-S-4FB	C6-succinimidyl 4-formylbenzoate
DFP	diisopropyl fluorophosphate
ECBC	U.S. Army Edgewood Chemical Biological Center
GD	pinacolyl methyl phosphonofluoridate, soman
OD	optical density
OPAA	organophosphorus acid anhydrolase
S-4FB	succinimidyl 4-formylbenzoate
S-HyNic	succinimidyl 6-hydrazinonicotinate acetone hydrazone
TEM	transmission electron microscope

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